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Target Gene Sequencing To Define the Susceptibility of *Neisseria meningitidis* to Ciprofloxacin

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Meningococcal *gyrA* gene sequence data, MICs, and mouse infection were used to define the ciprofloxacin breakpoint for *Neisseria meningitidis*. Residue T91 or D95 of GyrA was altered in all meningococcal isolates with MICs of ≥0.064 μg/ml but not among isolates with MICs of ≤0.032 μg/ml. Experimental infection of ciprofloxacin-treated mice showed slower bacterial clearance when GyrA was altered. These data suggest a MIC of ≥0.064 μg/ml as the ciprofloxacin breakpoint for meningococci and argue for the molecular detection of ciprofloxacin resistance.

Neisseria meningitidis is a Gram-negative encapsulated bacterium isolated only from humans, where it may provoke severe invasive infections (mainly septicemia and meningitis). Management of meningococcal disease requires prompt treatment of patients, as well as vaccination and/or chemoprophylaxis of contacts. The antibiotics currently recommended for chemoprophylaxis are rifampin, ciprofloxacin, and ceftriaxone (1). The emergence and expansion of meningococcal clones resistant to these antibiotics may jeopardize these recommendations. Ciprofloxacin resistance in meningococci was earlier linked to mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene (encodes subunit A of DNA gyrase) but no mutations in the QRDR of *gyrB*, *parC*, and *parE* (2). Here we correlate *gyrA* mutations and their *in vivo* impact on ciprofloxacin MICs for meningococcal clinical isolates.

This study examined all of the available meningococcal isolates collected from 1995 to 2011 with ciprofloxacin MICs of ≥0.064 μg/ml ($n = 19$) in four countries (France, Italy, Spain, and Sweden). Representative isolates with ciprofloxacin MICs of ≤0.032 μg/ml ($n = 177$) were also tested, as were two isolates of *N. gonorrhoeae* and *N. cinerea* with ciprofloxacin MICs of 0.250 and 0.125 μg/ml, respectively. Isolate typing was performed as previously described (3), and all those data are available at the *Neisseria*

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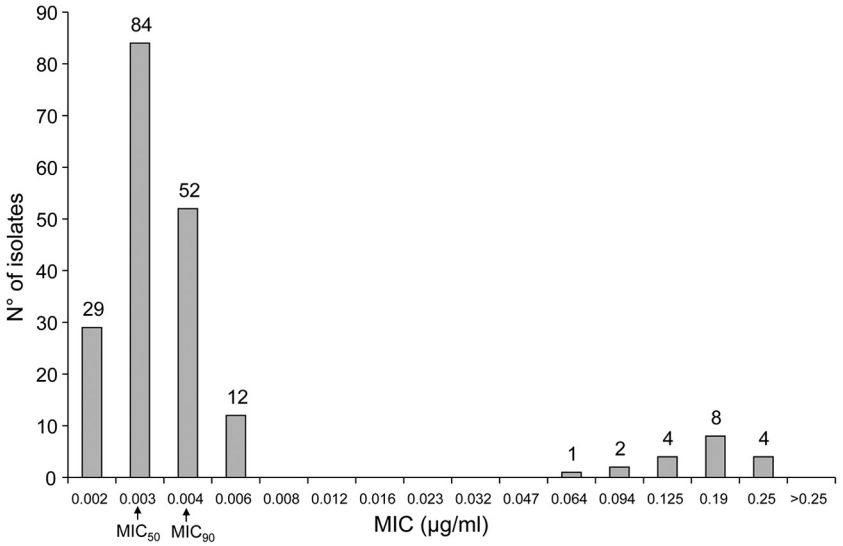

FIG 1 Ciprofloxacin MICs for the 177 meningococcal isolates with MICs of ≤0.032 μg/ml and the 19 with MICs of ≥0.064 μg/ml examined in this study. The number of isolates with each MIC is shown above each bar. The MIC_{50} and MIC_{90} are also indicated.

TABLE 1 Primers used in this study

Primer[a]	Sequence (5′–3′)[b]	Nucleotide position[c]
gyrA-1F	GTTTTCCCAGTCACGACGTTGTAATGACCGACGCAACCATCCGCCAC	1–847
gyrA-1R	TTGTGAGCGGATAACAATTTCCCAGCTTGGCTTTGTTGACCTGATAG	
parC-1F	GTTTTCCCAGTCACGACGTTGTAATGAATACGCAAGCGCACGCCCCA	1–822
parC-1R	TTGTGAGCGGATAACAATTTCGGAATTGGCGTTCGGCGGCAGCTC	
parE-1F	GTTTTCCCAGTCACGACGTTGTAGCCGAACTCGCCATCCGTCAG	1156–1600
parE-1R	TTGTGAGCGGATAACAATTTCGGGAAGTGGCGGTAGAACAGG	

[a] F and R stand for forward and reverse, respectively.
[b] The universal forward and reverse sequences (underlined) were added as adapters for sequencing.
[c] Positions are given according to the sequence of the corresponding genes of *N. meningitidis* strain MC58 (4).

PubMLST database (http://pubmlst.org/neisseria/). Both the 19 isolates with ciprofloxacin MICs of ≥0.064 μg/ml and the 177 isolates with ciprofloxacin MICs of ≤0.032 μg/ml belonged to different serogroups and to various clonal complexes. The distribution of ciprofloxacin MICs among the isolates tested is shown in Fig. 1.

Primers for the PCR amplification and sequencing of fragments including the QRDRs of *gyrA*, *parC*, and *parE* were designed (Table 1). Nine unique alleles were defined among the 177 isolates with MICs of ≤0.032 μg/ml (Table 2) with up to six polymorphic synonymous nucleotide sites. The corresponding isolates showed very low ciprofloxacin MICs that ranged from 0.002 to 0.006 μg/ml and were normally distributed around a median value of 0.003 μg/ml (Fig. 1). These alleles may therefore be defined as "wild-type" alleles. Sequencing identified eight different *gyrA* alleles among the 19 isolates with MICs of ≥0.064 (Table 2) and up to 35 polymorphic sites of which up to 6 were nonsynonymous and resulted in altered amino acid sequences in the GyrA QRDR at residue T91 or D95 (Table 2). Their geometric mean (95% confidence interval) ciprofloxacin MIC was 0.162 μg/ml (0.134 to 0.195 μg/ml), and their MICs ranged from 0.064 to 0.250 μg/ml. Moreover, no isolates with MICs between 0.008 and 0.032 μg/ml were characterized (Fig. 1). These data show that specific mutations in *gyrA* result in a significant MIC increase and allow the designation of a ciprofloxacin MIC of ≥0.064 μg/ml as the epidemiological cutoff value. No alterations in the QRDR of the *parC* or the *parE* gene were detected (data not shown). The identification of potential recombination events between two *gyrA* sequences was performed by using the START package available at http://pubmlst.org (5, 6). Significant putative recombination sites were detected in the *gyrA* alleles identified, suggesting a mosaic structure of *gyrA* and recombination between different *gyrA* alleles among *Neisseria* species.

The recombinant plasmid pDG34, which carries the bioluminescent *luxCDABE* operon under the control of the *porB* promoter (our unpublished data), was used to transform an *N. meningitidis* strain (clone 12) that is a serogroup C isolate with a ciprofloxacin MIC of 0.006 μg/ml and isogenic strain AS12 (ciprofloxacin MIC of 0.125 μg/ml), which harbors the most frequently altered *gyrA* allele (*gyrA8*, GyrA T91I alteration) (7, 8). Two transformants, clones 12lux (MIC of 0.006 μg/ml) and AS12lux (MIC of 0.125 μg/ml), were used to infect 8-week-old transgenic female mice expressing human transferrin by the intraperitoneal route (9). The protocol was approved by the Institut Pasteur Review Board, which is part of the Regional Committee of Ethics of Animal Experiments of the Paris region (permit 99-174).

Dynamic bioluminescence imaging (10) was performed 30 min after the injection of a bacterial suspension and showed sim-

TABLE 2 Characteristics and GyrA amino acid alterations of the *N. meningitidis* isolates tested in this study and the corresponding ciprofloxacin MICs

gyrA allele	No. of isolates	Country(ies)	MIC or MIC range (μg/ml) if >1 isolate	Geometric[a] mean MIC (μg/ml)	95% confidence interval	Alteration(s) in GyrA
1	24	France, Sweden	0.003–0.004	0.003	0.003–0.004	None
2	53	France, Italy, Sweden	0.002–0.006	0.003	0.003–0.003	None
3	7	France, Sweden	0.002–0.004	0.003	0.003–0.004	None
4	66	France, Italy, Sweden	0.002–0.006	0.003	0.003–0.004	None
5	13	France, Sweden	0.002–0.004	0.003	0.003–0.004	None
6	1	Italy	0.190	NA[c]	NA	T91I
7	4	France, Spain	0.125–0.250	0.162	0.134–0.195	T91I
8	7	France, Spain	0.125–0.250	0.175	0.139–0.221	T91I
9	2[b]	France	0.125–0.250	NA	NA	T91F, D95A
10	1	Spain	0.064	NA	NA	D95N
11	1	France	0.003	NA	NA	None
12	11	France, Italy, Sweden	0.002–0.004	0.003	0.003–0.004	None
13	3	France, Sweden	0.125–0.250	NA	NA	T91I
14	1	Spain	0.190	NA	NA	D95N
15	1	Spain	0.094	NA	NA	T91I
16	1	Spain	0.250	NA	NA	T91I
17	1	Sweden	0.002	NA	NA	None
18	1	Sweden	0.003	NA	NA	None

[a] Geometric mean MICs were determined for alleles shared by at least four isolates.
[b] The *gyrA9* allele was detected in two clinical isolates of *N. gonorrhoeae* and *N. cinerea*.
[c] NA, not applicable.

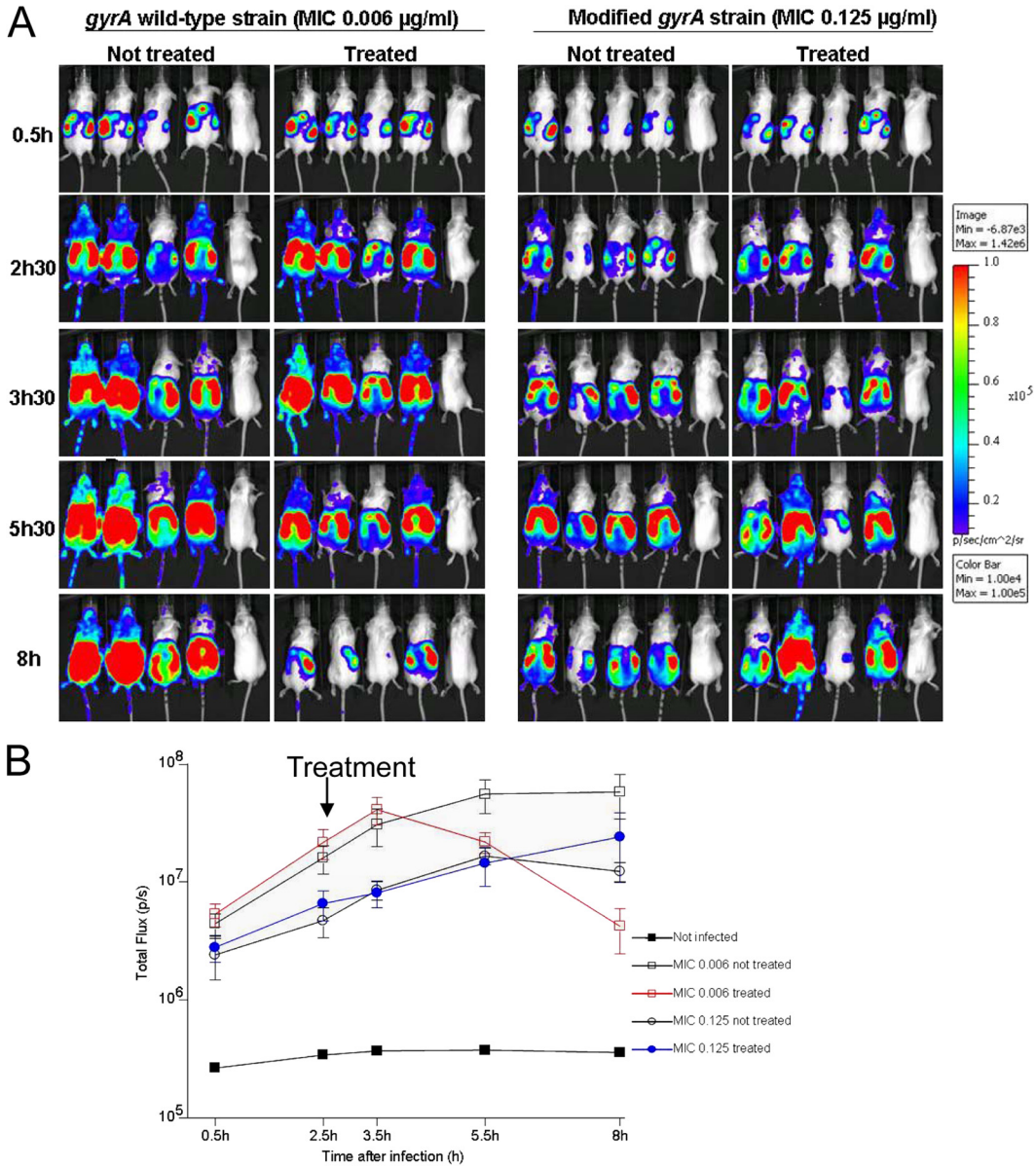

FIG 2 (A) Dissemination of *N. meningitidis* in BALB/c mice infected by the intraperitoneal injection of a bacterial suspension of *N. meningitidis* (5×10^6 CFU/ml). Clone 12lux (MIC of 0.006 μg/ml) and isogenic clone AS12lux (MIC of 0.125 μg/ml; GyrA T91I alteration) were used for infection. Two groups of four mice were infected with each strain. The fifth mouse in each group on the right is an uninfected control. One group of mice for each bacterial strain was treated with a single intramuscular injection of ciprofloxacin (5 mg/kg) after the acquisition of the images at 2.5 h. Mice were then analyzed for bioluminescence at the indicated times. Images (dorsal view) depict photographs overlaid with color representations of luminescence intensity, measured in photons per second and indicated on the scale at the right, where red is the most intense and blue is the least intense bioluminescence. (B) Bioluminescence was quantified and expressed as means ± standard deviations of four mice in each group at the indicated times by defining a specific representative region of interest encompassing the entire animal.

ilar levels of bioluminescence that increased after 2.5 h of infection in all of the mice. However, this increase was significantly more important in mice infected with the wild-type strain (clone 12lux) ($P = 0.005$), suggesting that mutation of *gyrA* has a biological cost. At 2.5 h, half of the mice infected with each strain were treated intramuscularly with a single 5-mg/kg dose (a total dose of 0.1 mg per 20-g mouse) of ciprofloxacin (Bayer) (11). At 5.5 and 8 h after infection (3 and 5.5 h after antibiotic treatment), the signal decreased significantly ($P < 0.05$) but only in ciprofloxacin-treated mice infected with clone 12lux (ciprofloxacin MIC of 0.006 μg/ml) (Fig. 2). Bioluminescent signals in mice that were infected with clone AS12lux (ciprofloxacin MIC of 0.125 μg/ml) continued to increase, regardless of ciprofloxacin treatment, suggesting that the strain is resistant to ciprofloxacin.

Meningococcal resistance to ciprofloxacin remains rare (2, 7, 12–15). Our current data show that alterations of the QRDR are still detected only in the *gyrA* gene and these alterations are associated with increased ciprofloxacin MICs. This differs from other bacterial species such as *N. gonorrhoeae*, where mutations of other target genes (*parC* and *parE*) are also detected (16). Only two residues in meningococcal GyrA seem to be associated with increased ciprofloxacin MICs (T91 and D95) that also result in cipro-

floxacin resistance in *N. gonorrhoeae*. Our results suggest that altered *gyrA* alleles may appear by single mutation or through interspecies recombination among isolates of different *Neisseria* species (14). The T91I mutation was associated with the persistence of bacteria in infected mice in spite of ciprofloxacin treatment. The T91I mutation in GyrA may be responsible for *in vivo* ciprofloxacin resistance. The use of this type of animal model may open new opportunities to test the *in vivo* phenotypes of isolates showing variable MICs.

A unique breakpoint (MIC, ≥0.064 μg/ml) for ciprofloxacin resistance may be suggested on the basis of *gyrA* sequence data correlated with MICs and *in vivo* data obtained with mice. Isolates resistant to ciprofloxacin (MIC, ≥0.064 μg/ml) belonged to different serogroups and different clonal complexes and showed different altered *gyrA* alleles. No clonal expansion with this ciprofloxacin resistance was detected, most likely because of the biological cost of *gyrA* mutations. Moreover, the ciprofloxacin-resistant isolates of serogroup A belonging to the ST-5 clonal complex showed different altered *gyrA* alleles, further arguing for independent selection. However, the detection of these isolates in Europe (where serogroup A is rare) may be due to imported infections that are most likely linked to recent travel to regions where serogroup A is endemic. Continuous phenotypic antibiotic resistance surveillance and rapid and reliable screening by a molecular method, as suggested in this study, are advocated.

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